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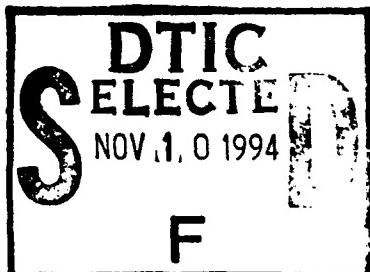


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**Investigation of laser-induced retinal damage:  
wavelength and pulselwidth dependent mechanisms**

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## **Investigation of laser-induced retinal damage: wavelength and pulsedwidth dependent mechanisms**

### **Scope**

The assessment of ocular laser injuries has historically been based on morphological or histological endpoints. Indeed, the current American National Standards Institute (ANSI) laser safety standard is set to one-tenth of the threshold laser exposure, at a given laser wavelength and exposure duration, that produces a minimum ophthalmoscopically-observable lesion in the retina of a test animal, usually a rhesus macaque. Although this approach has certainly been useful for screening large numbers of experimental animals and for being an assessment method applicable by laboratories without specialized facilities other than a standard ophthalmic slit lamp or fundus camera, the appearance of a minimal lesion in the retina (e.g. a just discernible spot) does not necessarily convey any information about the type of damage mechanism induced by the particular laser exposure. A morphological change may not be most sensitive indicator of a laser bioeffect. Permanent or transient changes at the cellular level may also occur, and must be detected by appropriate methods, particularly in view of emerging questions about the bioeffects produced by novel (e.g. ultrashort) laser systems, by extended or repeated exposures, and by low-level (biostimulatory) exposures.

For the present study, we proposed that laser bioeffects be sought in ocular tissue by specific biochemical assays, which would reflect the action of the laser energy at specific sites within the cell, at the cell membrane or in intracellular enzyme systems. We hypothesized that different laser exposure parameters could effect these biochemical assays differentially, by initiating tissue interactions through thermal, photochemical, or photodisruptive processes. Over the past three years of this project, we have made considerable progress in using biochemical assays to study photochemical or photooxidative processes. In addition, we have started to obtain data from assays designed to distinguish thermal from photooxidative and photodisruptive mechanisms. The current research addressing this issue uses cultured bovine RPE cells as the experimental model.

We shall briefly describe our experimental approach and then summarize the principal findings. At the end of the report, we shall list our publications and abstracts, summarize our presentations at scientific meetings, and list the collaborators who have helped in these experiments. We would also like to acknowledge the support of the Air Force Office of Scientific Research, without which this research would have not occurred, as well as the encouragement of the Program Managers in the Directorate of Chemistry and Life Sciences, initially Lt Col T. Jan Cerveny and presently Dr. Walter J. Kozumbo.

### **Introduction**

Although the consequences of excessive light exposure to the eye have been known since ancient times, the actual mechanisms of light damage in biological tissue have only been systematically investigated in this century. The response of tissue to laser or incoherent light depends on the power density, peak power, and wavelength of the irradiating energy. At least three light damage mechanisms have been identified. Photochemical damage is produced by short wavelength light (typically <550 nm) of long exposure duration, low peak power, and relatively low to moderate power density. Because tissue heating is minimal under these conditions, damage is

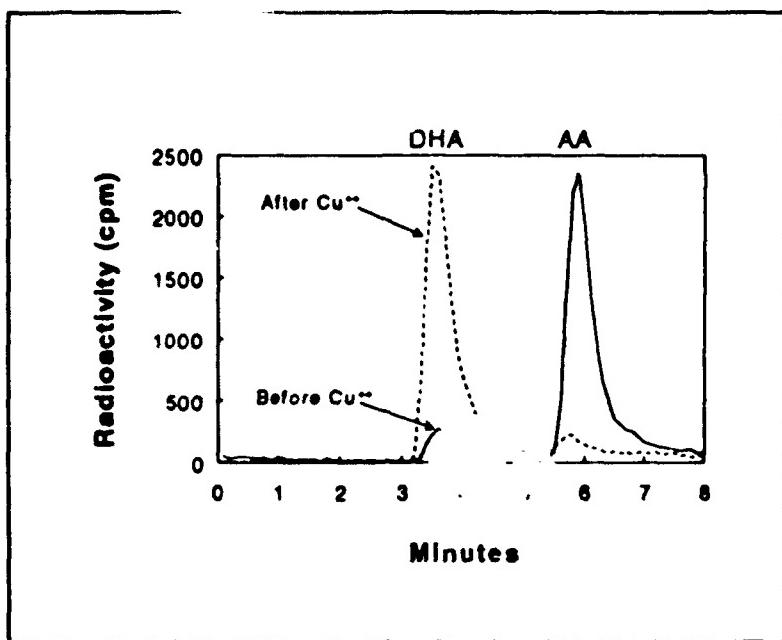
thought to occur as a result of excitation of target molecules to excited triplet states, some of which damage tissues directly through proton or electron transfers. The light-activated molecules may also cause damage indirectly by reacting with molecular oxygen to produce oxygen radicals, which are known agents of cellular damage. Thermal damage may be produced by light exposures of any wavelength capable of being absorbed by the tissue, given a sufficiently high power density and/or moderate to high peak power. Heating occurs by direct absorption of photons by a tissue chromophore which converts this photic energy into increased vibrational modes. The target chromophore, as well as surrounding structures depending on local heat conductivity, may then undergo thermal denaturation. At very high peak power, however, the strength of the E-, or electrical, field of the absorbed electromagnetic wave may exceed the dielectric properties of the absorbing tissue, causing optical breakdown, ionization, plasma formation, and other phenomena associated with nonlinear (photodisruptive) damage mechanisms.

The retinal pigment epithelium (RPE) has long been noted to be involved in the earliest stages of ocular light damage (15). This observation has raised the question of the extent of involvement of melanin in light damage. Melanin, because of its broadband absorption characteristics, is efficient at converting photic to vibrational mode energy (22), and is certainly involved in tissue damage due to thermal and non-linear mechanisms (15,16,29). Now, there is increasing evidence that melanin, which forms a free radical when exposed to UV and visible wavelengths (8,21), may also be able to promote photochemical tissue damage (5,11,12,14,24,28). We initiated our investigations with the observation that during visible light exposure, RPE melanin rapidly oxidized ascorbic acid (vitamin C). We considered it likely that this reaction resulted from the generation of free radicals during UVA and visible light exposure of the retina and RPE, and as such, could serve as a marker for photochemical damage. Based on this assumption, we investigated, during the past grant period, the exposure parameters and reaction conditions which optimized the light-activated oxidation of ascorbic acid by melanin. Further experiments conducted during this period defined the wavelength dependence of this reaction, its kinetics, and its specificity for ascorbic acid. We have also investigated other biochemical markers, such as the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) and the ions  $K^+$  and  $Ca^{+2}$  into the extracellular space, as possible methods to distinguish thermal and photochemical damage mechanisms. These investigations, while promising, have not progressed as far as the studies into free radical generation in ocular tissue.

#### Photooxidation of Ascorbic Acid in the Retinal Pigment Epithelium

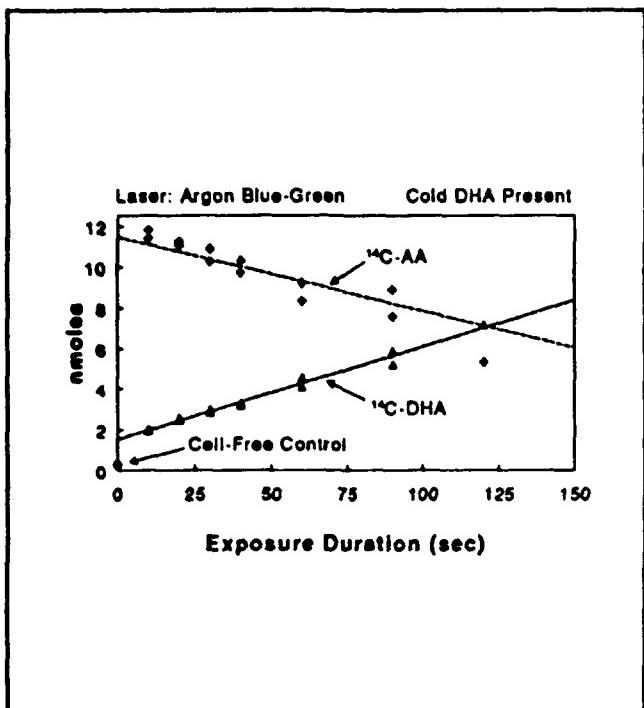
We have long had an interest in the metabolism of ascorbic acid in the eye. Under normal conditions, we found that ascorbic acid exists in the reduced form in the neural retina and aqueous humor, but mostly in the oxidized form (dehydroascorbic acid, or DHA) in the RPE and choroid (19,30). After a period of light stress, the amount of reduced ascorbic acid declined in the retina while DHA in the RPE and choroid increased. This pattern was found in guinea pig (30) and in the baboon (26). During the course of this investigation, we identified a possible mechanism for this loss of ascorbic acid, i.e. a rapid reaction between light-activated RPE melanin and ascorbic acid (13,14,20). Reduced and oxidized ascorbic acids can be quantitatively separated by HPLC on a  $\mu$ -Bondapak-NH<sub>2</sub> column eluted with ammonium phosphate. When [<sup>14</sup>C]-ascorbic acid is incubated with 0.4 mM CuSO<sub>4</sub>, the ascorbic acid is oxidized in a time-dependent fashion, and radioactivity is

progressively detected in the DHA peak. The complete separation of DHA and AA is shown in Figure 1.

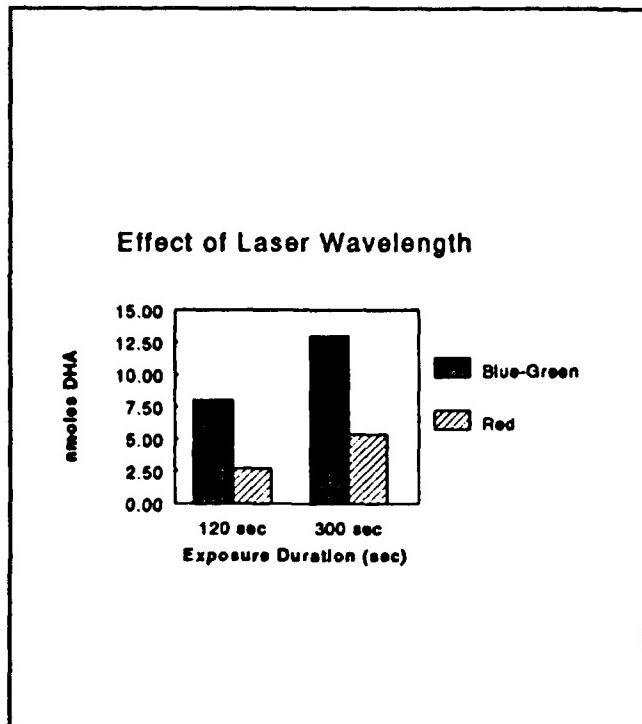


**Figure One.** Separation of AA and DHA by HPLC on  $\mu$ -Bondapak-NH<sub>2</sub> column, eluted with 20 mM ammonium phosphate at a flow rate of 1 ml/min. [<sup>14</sup>C]-AA was used, and the radioactivity in the eluate from the column was measured with a Radiomatic Beta-One flow-through beta counter. Oxidation of AA was achieved by incubation with 0.4 mM CuSO<sub>4</sub>. From Glickman & Lam, 1992 (ref. 13).

In double distilled water containing 0.2 mM EDTA, AA is quite stable and negligible autoxidation is observed. When whole, isolated bovine RPE cells, or melanin granules from the cells, are incubated with ascorbic acid, there is very slow oxidation of the AA, but if the mixture is exposed to visible laser (488.1 and 514.5 nm wavelengths), the ascorbic acid is rapidly oxidized to an extent proportional to the radiant exposure (Figure 2). The reaction is preferentially-driven by the shorter wavelengths, as indicated in Figure 3.



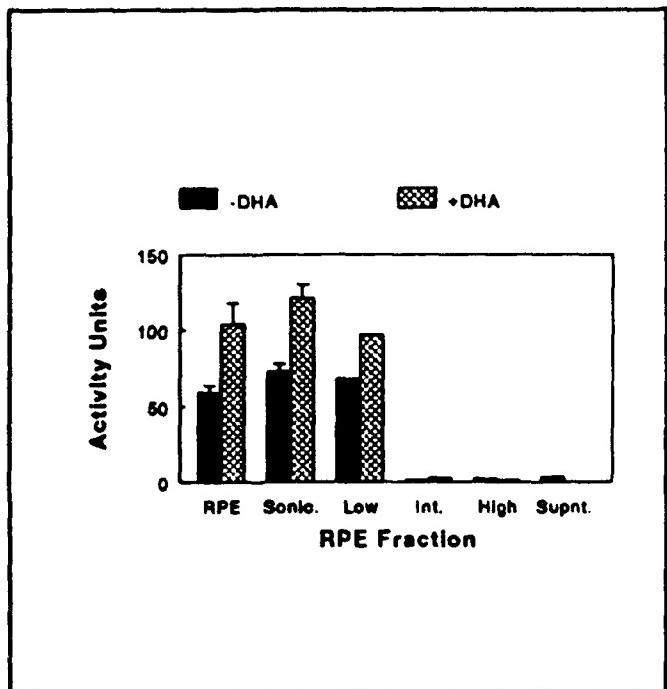
**Figure Two.** Effect of exposure duration on AA oxidation. Samples of the AA oxidation mixture containing 5 mM unlabelled DHA were exposed to the blue-green output of the argon laser for various durations at an irradiance of  $90 \pm 2 \text{ mW/cm}^2$ . The amount of AA oxidized was linear over the range of 10 to 120 sec. Linear functions were fitted to the AA and DHA data points. At each exposure duration, all the radioactivity was accounted for by the counts in the AA and DHA fractions, indicating no further reactions occurred, such as breakdown of DHA to  $\alpha$ -ketogulonic acid. From Glickman & Lam, 1992 (ref. 13).



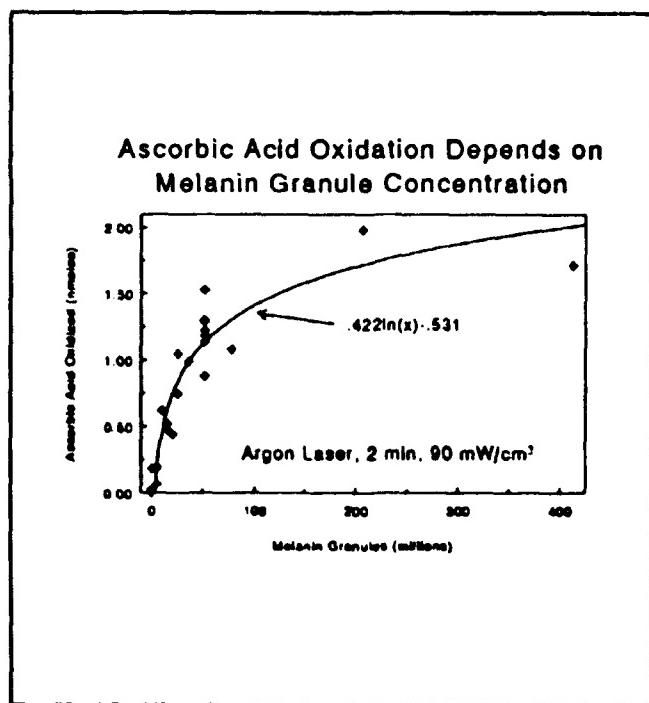
**Figure Three.** Effect of laser wavelength. Samples of the AA reaction mixture were exposed to either the argon blue green (488.1 and 514.5 nm mixed output) or the krypton red (647.1 nm) output at a sample irradiance of  $90 \pm 2 \text{ mW/cm}^2$  for either 120 or 300 sec. DHA production was analyzed by HPLC with subsequent measurement of [ $^{14}\text{C}$ ]-label in AA and DHA fractions. The shorter wavelength light was at least twice as effective in driving the melanin-activated oxidation reaction of AA. From Glickman & Lam, 1992 (ref. 13).

Sonicated RPE cells, as well as the cellular components precipitated by low speed centrifugation (fraction designated "Low" in Figure 4; see figure legend for experimental details), were able to catalyze the light-driven reaction. Examination of the low-speed pellet by light microscopy revealed that it was rich in melanin granules. Heating the cell fraction to  $80^\circ\text{C}$  did not destroy the light-induced activity. The heat-resistance of the activity is consistent with the hypothesis that melanin granules mediate the photosensitized oxidation reaction.

Melanin granules from bovine RPE cells were washed free of other cellular debris by successive centrifugation and resuspension in 0.3 M sucrose. These washed granules catalyzed the light-driven ascorbic acid oxidation to an extent dependent upon the number of melanin granules, when a saturating amount of AA is present (Figure 5).

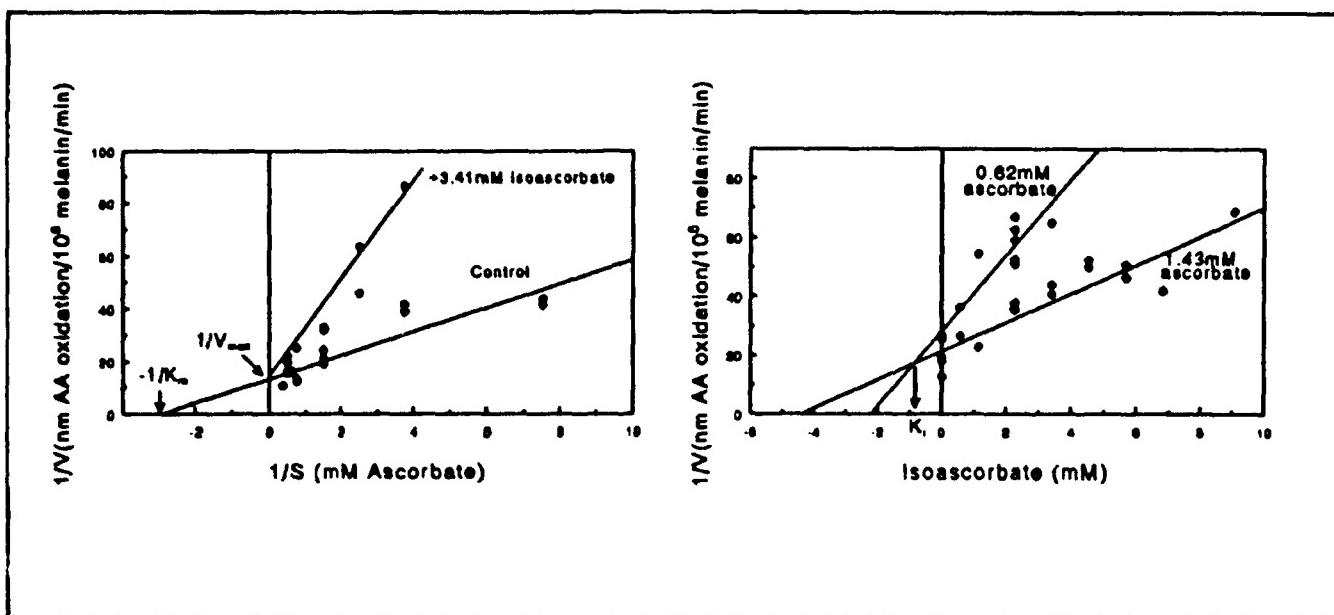


**Figure Four.** Relative activity of RPE cell fractions in catalyzing the light-activated oxidation of AA. AA oxidation during light exposure was measured with whole RPE cells ("RPE"), sonicated cells ("sonic"), the pellet resulting from centrifugation at 800 x g for 10 min ("Low"), the pellet resulting from centrifugation at 8,000 x g for 10 min ("Int"), the pellet resulting from centrifugation at 100,000 x g for 2 hr ("High"), and the final supernatant ("supnt"). Each fraction was exposed to the argon blue-green laser for 60 sec. From Glickman & Lam, 1992 (ref. 13).



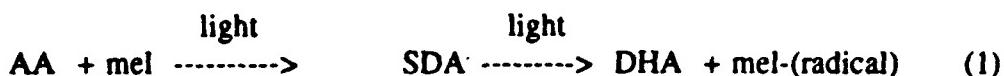
**Figure Five.** The dependence of AA oxidation on the concentration of melanin granules. From Lam & Glickman, 1992 (ref. 20).

The reaction follows first-order kinetics with a half-maximal rate occurring at about 0.33 mM ascorbic acid ( $K_m$ , Figure 6a). AA oxidation is competitively inhibited by isoascorbic acid, an epimer of ascorbic acid, with a  $K_i$  of 0.8 mM (Figure 6b).



**Figure Six.** A (left): Kinetics of light-activated melanin oxidation of AA, determined by Lineweaver-Burk analysis. B (right): Competition with ascorbic acid by isoascorbic acid.  $K_i$  determined by Dixon plot. From Glickman et al., 1993 (ref. 14).

The melanin may be capable of redox-cycling; in our experiments with radioactively-labelled ascorbic acid, we noted that when unlabelled DHA was added to the reaction mixture, there appeared to be increased light-activated oxidation of ascorbic acid. DHA, by itself, does not oxidize ascorbic acid. We hypothesize that this result could be due to redox-cycling of AA and DHA by melanin under the action of light, as outlined in the following reactions:



The reaction involves a 2-electron transfer through an intermediate, semidehydroascorbate radical (SDA). As light-driven AA oxidation (eqn. 1) occurs, labelled DHA is produced. As the activated melanin reduces DHA back to AA (eqn. 2), unlabelled DHA competes with the labelled pool, trapping more label in the DHA fraction, compared to a reaction mixture lacking any cold DHA in the initial conditions. Another possible mechanism for the DHA effect is that the presence of an excess of DHA minimizes the disproportionation of 2 SDA<sup>·</sup> into 1 AA and 1 DHA. The metal-catalyzed AA oxidation *in vitro* involves production of the unstable SDA<sup>·</sup> radical, which may undergo disproportionation (eqns. 3 and 4):

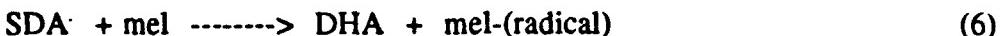




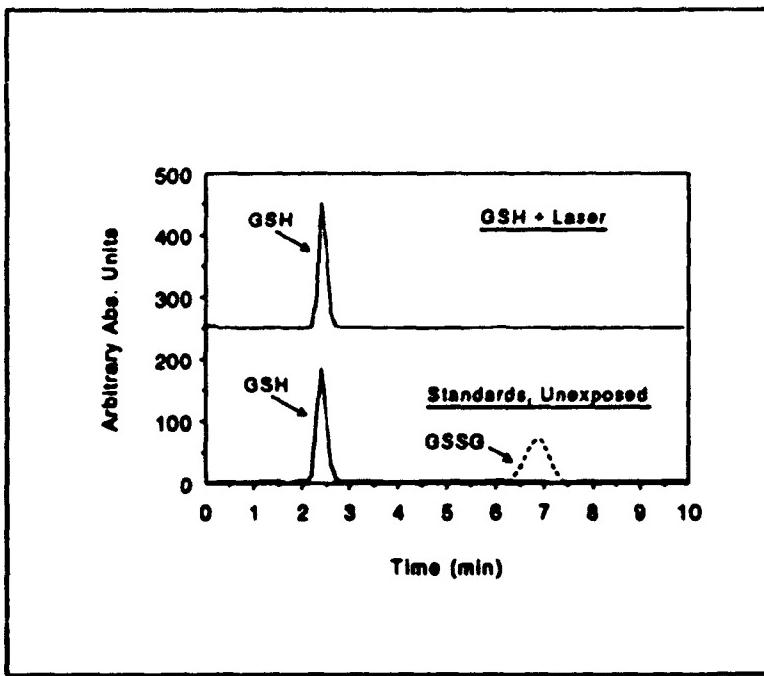
The rate of DHA formation is given by

$$\frac{dDHA}{dT} = k_1[SDA \cdot]^2 - k_2[AA][DHA] \quad (5)$$

so that if [DHA] is near 0, then the rate is given by  $k_1[SDA \cdot]^2$ , but if  $[DHA] \gg [SDA \cdot]$  then the rate approaches 0. Thus the disproportionation reaction is suppressed and SDA $\cdot$  loses the remaining electron to melanin resulting in conversion of all SDA $\cdot$  to DHA:



Interestingly, the reaction between melanin and ascorbic acid appears to be quite specific for ascorbic acid; other physiological antioxidants such as glutathione (20) either do not react with the melanin or react much more slowly, as shown in Fig. 7.



**Figure Seven.** Light-activated melanin fails to oxidize glutathione directly. Top: GSH and melanin granules exposed to argon blue-green laser. Bottom: GSH and GSSG standards separated on HPLC column. From Lam & Glickman, 1992 (ref. 20).

The reaction between light-activated melanin and ascorbic acid is rapid, with the rate proportional to the power density of the irradiating light (14). The reaction appears to cease as soon as the light or laser is turned off. Based on this observation, we proposed that the light-induced melanin radical reacts directly with ascorbic acid (13,14). There is the possibility, however, that the oxidation of ascorbic acid occurs through an oxygen radical intermediate. In an oxygenated environment,  $H_2O_2$  production has been reported during illumination of melanin, leading to the formation of hydroxyl radical (7). In our reaction system, we have found that under a nitrogen atmosphere, the reaction of light-activated melanin with ascorbic acid is reduced by about 15%, but not abolished, indicating that the melanin radical may have at least two types of reactive sites, one -- perhaps the majority

-- supporting Type I (direct) reactions, and the other supporting Type II reactions (involving an oxygen radical intermediate) (14).

#### Uptake of Ascorbic Acid by RPE cells.

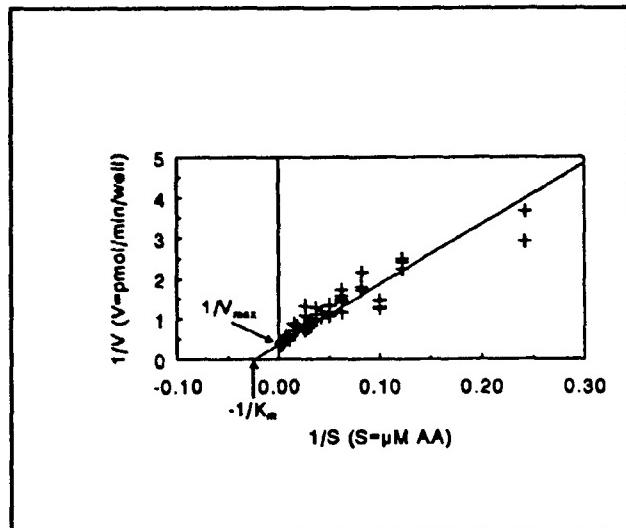
Ocular tissues have considerable ability to extract ascorbic acid from the circulation. Although the precise reason for the high concentration of ascorbic acid in the posterior segment of the eye is not known, it is possible that the ascorbic acid serves as an antioxidant to prevent photooxidative damage to sensitive retinal elements. In view of our finding that ascorbic acid is oxidized by light-induced melanin, and because the RPE regulates the passage of materials from the circulation to the retina, we determined ascorbic acid (AA) uptake into RPE (21). The transport of AA and its oxidized form, dehydro-L-ascorbic acid (DHA), was measured in cultured, SV-40 transformed, human RPE cells derived from the permanent RPE 28 SV4 line of the NIH Aging Cell Repository (kindly provided by Dr. Corrine G. Wong, of the Department of Ophthalmology, UC Irvine). Cells were cultured in Ham F12 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin-streptomycin, and fungizone. All cultures were grown in 96-well culture plates. Cells were initially seeded at a density of  $3.2 \times 10^4$  cells/ml. By one week, the cells had reached confluence at a density of 7,300 cells/mm<sup>2</sup>, yielding about  $2.3 \times 10^5$  cells/well.

For the measurement of AA uptake, the normal growth medium in each well was replaced with 0.1 ml of fresh medium containing [<sup>14</sup>C]-AA. In different experiments, unlabelled DHA, and various amounts of NaCl (to test the Na-dependence of the AA transporter), were also added. The cells were then incubated for 30 min at 37°C. At the end of the incubation period, the test medium was removed by suction, and the wells were rinsed four times with 0.25 ml of culture medium. Total cellular [<sup>14</sup>C]-AA was measured in cell extracts prepared with 0.25 ml of 0.2 N NaOH. From each extract, 0.2 ml was mixed with 5 ml of scintillation fluid and radioactivity was determined in an LKB model 1209 Rackbeta counter. Because labelled DHA was not available, the uptake of DHA into the RPE cells was estimated from the increase in intracellular AA (DHA was rapidly reduced to AA upon transport into RPE cells--see below). The AA content in these experiments was measured in cells extracted by 0.1 ml methanol, followed by measurement of the AA and DHA in a 10 µl aliquot of the extract. AA and DHA were separated by HPLC on a µ-Bondapak-NH<sub>2</sub> column eluted with 20 mM ammonium phosphate, with detection of the eluate at 265 nm.

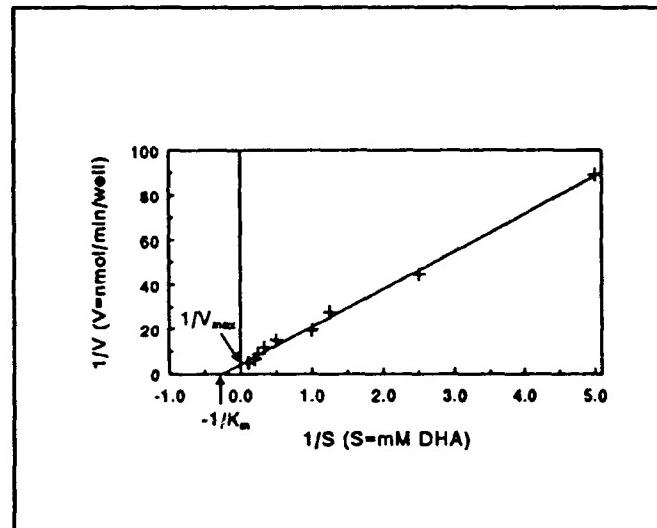
The uptake of labelled AA exhibited first order saturation kinetics as shown in Figure 8. The rate of uptake was dependent on the AA concentration in the culture medium and reached a maximum value of 2.74 pmol/min/well. The uptake of AA was sodium dependent and was maximal when the NaCl concentration was increased above 100 mM. The DHA transporter was also found to be Na-dependent, and exhibited first order kinetics (see Figure 9). Because negligible DHA was found inside the RPE cells, and the intracellular content of AA increased when the cells were incubated in medium containing DHA, the cells were evidently able to reduce DHA to AA after uptake. The reduction of DHA to AA required the active metabolism of the living cell, because RPE cell homogenates did not reduce DHA.

DHA did not compete with AA for transport into RPE cells. The noncompetitive relationship of AA and DHA was another indicator that different transporters were responsible for their uptake into the cell. As shown in Table 1, the measured kinetics of the AA and DHA transporters differed markedly, and indicated that the specificity of the DHA transporter was much lower than that of the

AA transporter. These data indicate that AA enters the RPE cell by a transporter of high specificity (low  $K_m$ ) but relatively modest maximum rate (moderate  $V_{max}$ ). In contrast, DHA is taken up by the RPE cell by a relatively non-specific transporter (high  $K_m$ ) with a high maximum velocity (high  $V_{max}$ ). It is to be noted that the  $K_m$  of the AA transporter is similar to the normal concentration of AA in the serum (0.01 - 0.06 mM), so that under normal physiological conditions, the AA transporter is near saturation. Thus, because there is little DHA normally found in the blood, the DHA transporter is probably far from saturation, and any excess DHA produced in the vicinity of the RPE is likely to be rapidly taken up by the RPE cells and recycled back to AA.



**Figure 8.** Concentration-dependent AA uptake. The cells were incubated in complete culture medium with specified concentrations of [<sup>14</sup>C]-AA for 30 min. The cells were then washed and extracted by NaOH to determine the radioactivity taken up into the cells. Plus symbols represent individual determinations; data are plotted on double reciprocal axes and a linear function has been fitted to the data for a Lineweaver-Burk analysis of kinetic data. From Lam et al., 1993 (ref. 21).



**Figure 9.** Concentration-dependent DHA uptake. The cells were incubated in complete culture medium containing different concentrations of DHA for 30 min. The cells were washed, extracted in methanol, and the AA concentration in the methanol extract was determined by HPLC as described in the text. Data plotted as in Figure 8 for Lineweaver-Burk kinetic analysis. From Lam et al., 1993 (ref. 21).

**Table 1.** Kinetic properties of ascorbic and dehydroascorbic acid uptake.

Substrate	$K_m$ , mM	$V_{max}$ , pmol/min/well
DHA	$5.670 \pm 0.290$	$325.5 \pm 8.4$
AA	$0.041 \pm 0.003$	$2.7 \pm 0.1$

Data from Lam et al., 1993 (ref. 21).

## Other measures of cellular responses to laser radiation

### Ionic Changes.

In addition to the photochemical changes associated with laser exposures, we have investigated other measures of cellular responses to laser irradiation. Most of these measures are based on the loss of intracellular contents into the extracellular space because of laser-induced damage to the cell's plasma membrane. The first of these measures we investigated was loss of intracellular K<sup>+</sup> ions due either to damage to the ATP-dependent Na<sup>+</sup>-K<sup>+</sup> ion exchange pumps, allowing progressive leakage of K<sup>+</sup> ions out of the cell, or to actual leaks produced in the plasma membrane by the deposition of laser energy.

Experiments were conducted in RPE cells isolated from bovine eyes and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose. Cells were used immediately following isolation and were not cultured further, so additional nutrients were not added to the medium. Suspensions of cells in 100 µl were placed in plastic microcentrifuge tubes and exposed to the mixed, 514.5 and 488.1 nm output of the argon CW laser for 30 seconds at various fluences (Table 2). Following the exposure, the centrifuge tubes were spun at 2000 rpm for 2 minutes and a cell-free, 5 µl aliquot was taken from the supernatant, diluted with 0.5 ml of HPLC-grade water, and run on a Waters 4000 High Performance Capillary Electrophoresis (HPCE) unit with cation analysis protocol. Cations were quantified in millimoles based on their peak area in the chromatogram. Some sample results are shown in Table 2 (right).

The results suggest that at the highest fluence used, 2.4 W/cm<sup>2</sup>, [K<sup>+</sup>] was increased in the medium. However, the metric we had hoped to use in these experiments, the ratio of [Na<sup>+</sup>]/[K<sup>+</sup>], was not decreased compared to Control, because [Na<sup>+</sup>] was also increased. This change in extracellular ions appeared to be inconsistent across several experiments and we concluded that the total change in extracellular ions produced by a mild thermal stress was so small that it may have been confounded by experimental errors, such as pipetting errors in the small samples or similar factors. Therefore, we decided to look at another measure, the leakage of lactate dehydrogenase from the cytoplasm of exposed cells.

**Table 2.** Isolated bovine RPE cells suspended in DMEM were exposed to the output of an argon, CW laser for 30 sec at the fluences indicated. "Medium": DMEM only (no cells present); "Control": Unexposed RPE cells in DMEM; the other experiments were RPE cells in DMEM exposed to the laser at the indicated fluences. Analysis details are given in the text.

### Effect of Laser Exposure on Extracellular Na<sup>+</sup> and K<sup>+</sup>

Expt.	Na <sup>+</sup> (mM)	K <sup>+</sup> (mM)	Na <sup>+</sup> /K <sup>+</sup>
Medium	159	4.2	38.0
Control	138	4.9	28.2
1.1 W/cm <sup>2</sup>	166	4.6	36.0
1.7 W/cm <sup>2</sup>	164	4.9	33.6
2.4 W/cm <sup>2</sup>	178	5.4	32.8

## Lactate Dehydrogenase (LDH) assay for laser-induced cellular damage.

The rationale for this assay is that LDH is normally contained in the cytoplasm of cells and should not be present in significant quantities in the extracellular space. The enzyme, being a large molecule, would only be able to escape the cell if the plasma membrane were broken or disrupted by the external stressor. Thus, an increase in the extracellular LDH activity level could serve as an indicator of plasma membrane integrity. The assay for LDH is straightforward; it is based on the catalysis by LDH of the conversion of lactic acid to pyruvate with the concomitant reduction of nicotinamide adenine dinucleotide (the conversion of NAD to NADH). The increase in the amount of NADH is monitored spectrophotically by the increase in optical density at 340 nm. The rate of the increase in the absorbance at 340 nm is correlated to the amount of LDH present, and thus can be used as a quantitative assay for LDH.

These experiments were done in RPE cells isolated from 6 bovine eyes and separated into pigmented (from the inferior part of the globe) and non-pigmented (from the superior part of the globe) populations. RPE cells in the superior portion of the eye contain few melanin granules compared to cells in the inferior portion. The cells were washed once in DMEM, resuspended into a volume of 1 ml, and divided into 50  $\mu$ l aliquots. Each aliquot was exposed to the mixed output of the argon laser for 30 sec at various fluences as indicated in Figure 10.

There is an indication that the efflux of LDH is increased above radiant exposures of 1 W/cm<sup>2</sup>, but at 2.7 W/cm<sup>2</sup>, LDH activity was reduced below control levels. Noticeable heating of the sample occurred at this irradiance, so that thermal denaturation of the enzyme is a possible explanation for this observation. Also, the "pigmented" cells released markedly higher amounts of LDH than did the non-pigmented cells. These results support the role of melanin in mediating thermal cellular stress, owing to its broadband absorption of optical energy.

These experiments indicated that an LDH assay holds promise as a cellular assay for laser damage. Currently, a master's level student is working on this assay, refining the exposure conditions, as well as the cell culture conditions for the RPE cell lines. The goal of the project is to compare the effects of CW laser exposures, producing primarily thermal damage, to the effects of exposures of a Q-switched laser delivering the same total energy at approximately the same wavelength, but with the photons arriving in a short pulse or train of pulses lasting, at most, several nanoseconds. This latter type of exposure is expected to induce not only thermal stress, but also photoacoustic (mechanical) damage and possibly optical breakdown in the tissue. We hypothesize

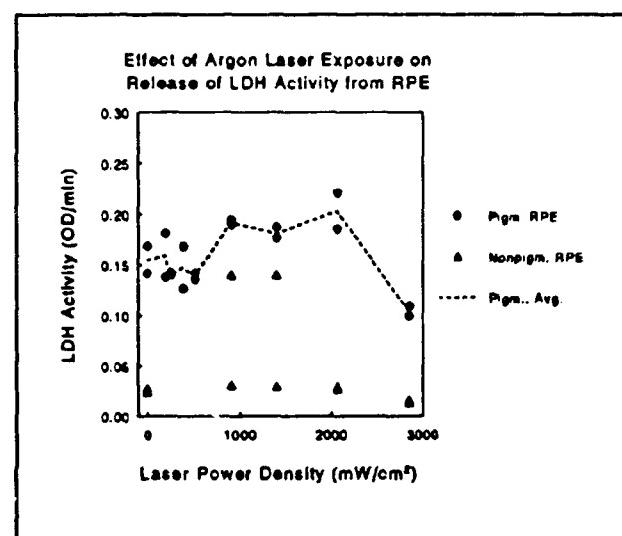


Figure 10. LDH activity released from RPE cells exposed to the mixed 488.1 + 514.5 nm output of an argon laser for 30 sec at various fluences. "Pigmented" cells were obtained from the inferior portion of bovine eyes, while the "non-pigmented" cells were obtained from the superior portion. Dotted line is the calculated sample mean at each of the exposure levels. LDH assay performed as described in the text.

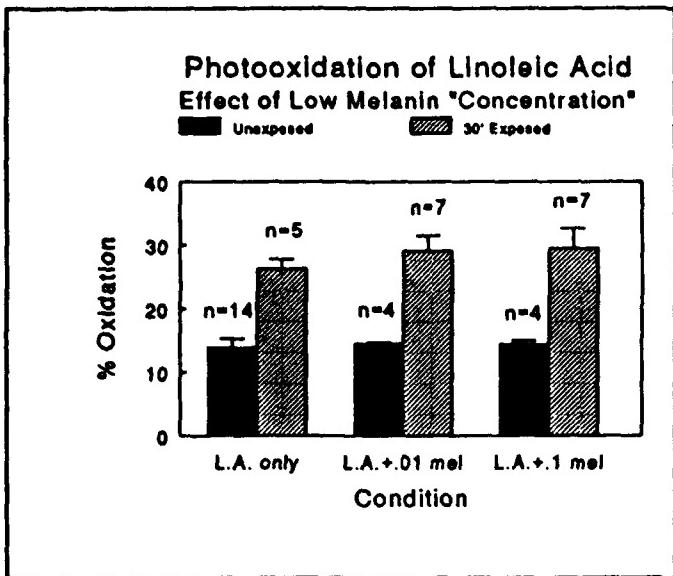
that the resulting disruption of plasma membranes could conceivably result in a larger and more rapid release of LDH into the extracellular space than occurs after simple thermal stress. This study should be completed by the end of this year.

#### Other reactions of light-activated melanin.

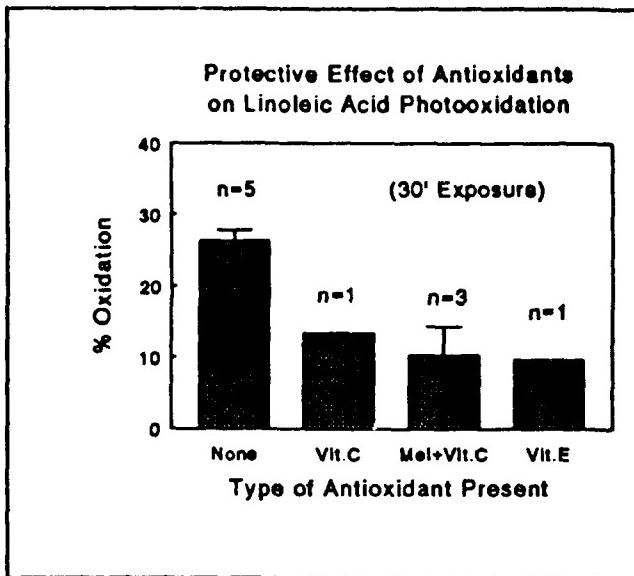
#### Melanin and the Photooxidation of Linoleic Acid.

The reactivity of light-activated melanin towards ascorbic acid raises the important question of its reactivity to other cellular components. If the melanin radical, *in vitro*, can be shown to react with other cellular components such as fatty acids, proteins, and nucleic acids, then the involvement of melanin, *in vivo*, in photochemical damage must be considered. To begin our investigation of other light-activated melanin reactions, we are determining the interaction of melanin and linoleic acid, a polyunsaturated fatty acid. For these experiments, linoleic acid (Sigma Chemical) and RPE melanin granules isolated from bovine eyes are used. Photooxidation of linoleic acid is produced by exposure to the broadband output of a 150W xenon arc lamp. Photochemical reactions are conducted in an enclosed glass vessel so that temperature and atmospheric conditions can be controlled. Native linoleic acid is separated from its hydroperoxides by HPLC on a Delta PAK-C<sub>18</sub> column, eluted with a 88% acetonitrile-12% water-0.01% trifluoroacetic acid mixture. Linoleic acid is detected by absorbance at 201 nm, while hydroperoxides of linoleic acid are determined by absorbance at 232 nm due to the presence of conjugated double bonds.

Exposure of the linoleic acid alone to the xenon arc lamp results in production of linoleic hydroperoxides (LHP). This photochemical reaction is temperature dependent; for a 30' exposure producing a sample irradiance of 215 mW/cm<sup>2</sup>, the amount of LHP produced doubles as the reaction temperature is raised from 0° to 80°C. The photochemical production of LHP was not entirely dependent on the presence of oxygen in the reaction chamber. Under about 600 mm Hg of vacuum, or under a N<sub>2</sub> atmosphere, the production of LHP was reduced about one third from that produced under normal laboratory conditions. When melanin was introduced into the reaction mixture, its effect on LHP production was "concentration" dependent, i.e. dependent on the number of granules present. At high granule counts (approximately 10<sup>8</sup> granules/ml), the production of LHP was reduced, probably because of light absorption and self-screening by the melanin. At lower counts ( $\leq 10^7$  granules/ml), the production of LHP was enhanced by up to 20% (Figure 11). This increase in LPH production was significant ( $p < .05$ , 2-tailed t-test). As shown in Figure 12, the photooxidation of linoleic acid was reduced by ascorbic acid (Vitamin C) or  $\alpha$ -tocopherol (Vitamin E), regardless of the presence or absence of melanin granules. These observations suggest that the melanin radical, at least under certain conditions, may promote lipid peroxidation, thereby contributing to photochemical damage. The antioxidant status of the tissue obviously plays an important role in modulating the reactivity of melanin with other cellular components. These preliminary observations support a further investigation of the reactions of light-activated melanin, and the conditions under which they occur.



**Figure 11.** The presence of melanin granules at low "concentration" enhances the photooxidation of linoleic acid. L.A. = linoleic acid; 0.1 mel and 0.01 mel refer to fold-dilutions of the stock suspension of melanin granules which contained  $2 \times 10^8$  granules/ml.



**Figure 12.** Photooxidation of linoleic acid is prevented by the presence of Vitamin C (2.3 mM) or E (2.4 mM) in the reaction mixture.

### Conclusions

We have gathered data showing that, when exposed to light or laser radiation, melanin is capable of directly oxidizing suitable substrates. The reaction proceeds in an irradiance- and wavelength-dependent fashion, and thus appears to be suitable as an indicator of photooxidative stress produced in pigmented tissue by the action of laser light. The experimental evidence obtained to date indicates that melanin oxidizes the ascorbic acid by a free radical mechanism, and is consistent with the hypothesis that melanin may serve as a photosensitizer. The available data also indicate that the light-driven melanin reactions proceed through Type I (direct interaction) and Type II (via an oxygen radical intermediate) mechanisms. A reasonable explanation for this finding is that there are at least two different reactive sites induced on the melanin heteropolymer by light. Thus, because ascorbic acid is readily oxidized at both sites during light exposure, the melanin-ascorbic acid oxidation reaction serves as a reasonable assay for photochemical (free radical) stress induced in ocular tissue during laser and light exposure.

We further hypothesize that ocular melanin plays a dual role in phototoxicity, i.e. it may mediate both thermal and photochemical light-tissue interactions. It has been determined that up to 50% of light entering the eye is absorbed in the RPE and choroid (6). Obviously, the melanin situated in these layers serves as a prime absorber of these "excess" photons. There must be a mechanism for channelling away from sensitive structures the excess light energy. One possible way is by conversion of the light energy into heat (23). Another alternative is that, if sufficient electrons are excited to triplet states (or singlets in the case of O<sub>2</sub>) by the absorbed radiation (as is likely with short-wavelength light), then energy will be transferred *in toto* to an acceptor molecule. We propose

that the light-activated melanin normally transfers its absorbed photonic energy via an excited free radical state to ascorbic acid, and thereby is prevented from reacting with other cellular components.

The oxidized ascorbic acid (dehydro-L-ascorbic acid) may then be recycled, which restores the supply of available ascorbic acid and channels the surplus energy derived from photons (not used for the visual process) down a chain of coupled redox reactions. Although the enzymatic mechanism for recycling oxidized ascorbic acid has not been fully characterized (24), it is clear that ascorbic acid undergoes redox cycling with other antioxidants and enzyme systems in the cell. As long as the supply of reduced ascorbic acid is adequate, damaging photooxidative reactions are prevented. But if the light insult is sufficiently intense or prolonged, or if the supply of antioxidants is compromised, then the possibility exists that the activated melanin could initiate oxidative reactions, e.g. lipid peroxidation in the photoreceptor outer segments. This may become more likely in age, either because of a loss of normally functioning melanin (10) or because of a progressive loss in the ability to mobilize ascorbic acid. Therefore a decline in the redox state of the aging eye may result in melanin acting as a photosensitizer instead of a protective agent. Indeed, our current work indicates that in the absence of vitamin C or E, dilute suspensions of melanin granules mixed with linoleic acid promote the production of linoleic hydroperoxides during light exposure. Thus melanin may serve as both sensitizer and protector, depending on the physiological state of the eye.

The other assays of cellular responses to laser and light exposure that have been examined, i.e. changes in extracellular ionic balance and release of LDH into the extracellular space, are less well developed, although the LDH assay shows some promise for distinguishing photodisruptive from purely thermal damage mechanisms. LDH release from cells has been used to demonstrate free radical (photodynamic) stress induced by the action of laser-activated photosensitizers in cultured cells (2,3). On the other hand, the ion assay appears to be impractical for routine use, because the number of ions released from cells by a given exposure are probably too small for reliable detection against the normal ionic concentrations of the cellular environment. Further development of cellular assays for light damage may productively utilize detection of various components of the "stress response" of mammalian cells (18), which is exhibited after exposure to various stressors including radiation (28), UV (9,26), oxidative stress (4,17), and activating agents such as phorbol esters (1,9). Because the stress response involves production of novel RNA messengers, proteins, or protein modifications, sensitive immunobiological and molecular probes may be used to detect the onset of cellular stress, and therefore provide the most accurate detection yet of threshold cellular changes after laser exposure.

#### References cited

1. Angel P, Karin M. The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. *Biochim Biophys Acta* 1991;1072:129-57.
2. Artuc M, Ramshad M, Kappus H. Studies on acute toxic effects to keratinocytes induced by hematoporphyrin derivatives and laser light. *Arch Dermatol Res* 1989;281:491-4.
3. Artuc M, Ramshad M, Reinhold C, Kappus H. DNA damage caused by laser light activated hematoporphyrin derivatives in isolated nuclei of human melanoma cells. *Arch Toxicol, Suppl* 1988;12:56-9.

4. Bauskin AR, Alkalay I, Ben-Neriah Y. Redox regulation of a protein tyrosine kinase in the endoplasmic reticulum. *Cell* 1991;66:686-96.
5. Black HS. Potential involvement of free radical reactions in ultraviolet light-mediated cutaneous damage. *Photochem Photobiol* 1987;46:213-21.
6. Boettner EA, Wolter JR. Transmission of the ocular media. *Invest Ophthalmol Vis Sci* 1962;1:776-83.
7. Burke JM, Korytowski W, Sarna T. *In vitro* photooxidation of RPE melanin; Role of hydrogen peroxide and hydroxyl radicals. *Photochem Photobiol* 1991;53(Suppl):15S.
8. Cope FW, Sever RJ, Polis BD. Reversible free radical generation in the melanin granules of the eye by visible light. *Arch Biochem* 1963;100:171-7.
9. Devary Y, Gottlieb RA, Smeal T, Karin M. The mammalian ultraviolet response is triggered by activation of src tyrosine kinases. *Cell* 1992;71:1081-91.
10. Feeney-Burns L, Hilderbrand ES, Eldridge S. Aging human RPE: morphometric analysis of macular, equatorial and peripheral cells. *Invest Ophthalmol Vis Sci* 1984;25:195-200.
11. Felix CC, Hyde JS, Sarna T, Sealy RC. Melanin photoreactions in aerated media: electron spin resonance evidence for production of superoxide and hydrogen peroxide. *Biochem Biophys Res Commun* 1978;84:335-41.
12. Felix CC, Hyde JS, Sealy RC. Photoreactions of melanin: a new transient species and evidence for triplet state involvement. *Biochem Biophys Res Commun* 1979;88:456-61.
13. Glickman RD, Lam K-W. Oxidation of ascorbic acid as an indicator of photooxidative stress in the eye. *Photochem Photobiol* 1992;55:191-6.
14. Glickman RD, Sowell R, Lam K-W. Kinetic properties of light-dependent ascorbic acid oxidation by melanin. *Free Rad Biol Med* 1993;15:453-7.
15. Ham Jr WT, Allen RG, Feeney-Burns L, et al. The involvement of the retinal pigment epithelium (RPE). In: Waxler M, Hitchins VM, eds. *Optical Radiation and Visual Health*. Boca Raton, FL: CRC Press, 1986:43-67.
16. Ham Jr WT, Mueller HA, Ruffolo JJ, Guerry D, Guerry RK. Action spectrum for retinal injury from near-ultraviolet radiation in the aphakic monkey. *Am J Ophthalmol* 1982;93:299-306.
17. Heffetz D, Zick Y.  $H_2O_2$  potentiates phosphorylation of novel putative substrates for the insulin receptor kinase in intact Fao Cells. *J Biol Chem* 1989;264:10126-32.
18. Herrlich P, Angel P, Rahmsdorf HJ, et al. The mammalian genetic stress response. *Adv Enzyme Regul* 1985;25:485-504.

19. Lai Y-L, Fong D, Lam K-W, Wang H-M, Tsin ATC. Distribution of ascorbate in the retina, subretinal fluid and pigment epithelium. *Curr Eye Res* 1986;5:933-8.
20. Lam K-W, Glickman RD. Prevention of light-induced free radical production from melanin granules by ascorbic acid. In: Yagi K, Niki E, Yoshikawa T, eds. *Oxygen Radicals*. Amsterdam: Elsevier Science Publishers, 1992:633-6.
21. Lam K-W, Yu H-S, Glickman RD, Lin T. Sodium-dependent ascorbic and dehydroascorbic acid uptake by SV-40-transformed retinal pigment epithelial cells. *Ophthal Res* 1993;25:100-7.
22. Mason HS, Ingram DJE, Allen B. The free radical property of melanins. *Arch Biochem Biophys* 1960;86:225-30.
23. McGinness J, Proctor P. The importance of the fact that melanin is black. *J Theoret B* 1973;39:677-8.
24. Rose RC, Bode AM. Tissue-mediated regeneration of ascorbic acid: Is the process enzymatic? *Enzyme* 1992;46:196-203.
25. Sarna T. Properties and function of the ocular melanin - A photobiophysical view. *J Photochem Photobiol B* 1992;12:215-58.
26. Schieven GL, Kiriha JM, Gilliland LK, Uckun FM, Ledbetter JA. Ultraviolet radiation rapidly induces tyrosine phosphorylation and calcium signalling in lymphocytes. *Mol Biol Cell* 1993;4:523-30.
27. Tso MOM, Woodford BJ, Lam K-W. Distribution of ascorbate in normal primate retina and after photic injury: a biochemical, morphological correlated study. *Curr Eye Res* 1984;3:181-91.
28. Uckun RM, Schieven GL, Tuel-Ahlgren LM, et al. Tyrosine phosphorylation is a mandatory proximal step in radiation-induced activation of the protein kinase C signaling pathway in human B-lymphocyte precursors. *Proc Natl Acad Sci USA* 1993;90:252-6.
29. Weiter JJ. Phototoxic changes in the retina. In: Miller D, ed. *Clinical Light Damage to the Eye*. New York: Springer-Verlag, 1987:79-125.
30. Wolbarsht ML, Walsh AW, George G. Melanin, a unique biological absorber. *Appl Opt* 1981;20:2184-6.
31. Woodford BJ, Tso MOM, Lam K-W. Reduced and oxidized ascorbates in guinea pig retina under normal and light-exposed conditions. *Invest Ophthalmol Vis Sci* 1983;24:862-7.

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(Articles)

Glickman RD and Lam K-W. Oxidation of ascorbic acid as an indicator of photooxidative stress in the eye. *Photochem Photobiol* 55:191-196, 1992.

Lam K-W and Glickman RD. Prevention of light-induced free radical production from melanin granules by ascorbic acid. In: Yagi, K; Kondo, M, Niki, E, and Yoshikawa, T, eds. *Oxygen Radicals*. Amsterdam: Elsevier Science Publishers; 1992:633-636.

Lam K-W, Yu HS, Glickman RD, and Lin T. Sodium dependent ascorbic and dehydroascorbic acid uptake by SV-40 transformed pigment epithelium cells. *Ophthalmic Res* 25:100-107, 1993.

Glickman RD, Sowell R, and Lam K-W. Kinetic properties of light-dependent ascorbic acid oxidation by melanin. *Free Rad Biol Med* 15:453-457, 1993.

(Abstracts and Meeting Presentations)

Lam K-W and Glickman RD. Oxidation-reduction cycling of ascorbate in the posterior segment of the eye. *Invest Ophthal Vis Sci* 32(ARVO suppl.):1101, 1991. (Presented at the Association for Research in Vision and Ophthalmology Annual Meeting, 28 April - 3 May, 1991, Sarasota, FL).

Glickman RD and Lam KW. The role of melanin in photooxidation of ocular ascorbic acid. *Photochem Photobiol* 53 (Work in Progress supplement), 1991. (Presented at the American Society for Photobiology 19th Annual Meeting, 22-26 June, 1991, San Antonio, TX).

Lam K-W, Yu HS, Glickman RD, and Lin T. Mechanism of ascorbic acid uptake into SV-40 transformed retinal pigment epithelial cells. *Invest Ophthal Vis Sci* 33(ARVO Suppl.):921, 1992. (Presented at the Association for Research in Vision and Ophthalmology Annual Meeting, 3-8 May, 1992, Sarasota, FL).

Glickman RD, Lam K-W, Yu HS. Reduction of photooxidative stress in the retina by uptake of dehydro-L-ascorbic acid. *Exp Eye Res* 55:(ICER suppl):248, 1992. (Presented at the International Congress of Eye Research, 20-25 Sept, 1992, Stresa, Italy).

Lam K-W, Wang L, and Olson RE. An organoselenium compound in the bovine retina with phospholipid hydroperoxidase (PHGPX) activity. (Presented at the Int. Conf. on Critical Aspects of Free Radicals in Chemistry, Biochemistry, and Medicine. Sponsored by the Soc. for Free Radical Research - Europe, 14-17 Feb, 1993, Vienna, Austria).

Glickman RD and Lam KW. Light, photosensitizers and oxygen radicals: How are they involved in retinal light damage? *Soc Neurosci Abstr* 19 (part 1):841, 1993. (Presented at the Society for Neuroscience Annual Meeting, 7-12 Nov, 1993, Washington, D.C.).

Glickman Rd and Lam K-W. The interaction of RPE melanin and ascorbic acid with the photooxidation of linoleic acid. *Free Rad Biol Med* 15:529, 1993. (Presented at Oxygen '93, 12-17 Nov, 1993, Charleston, SC).

Glickman RD, Sowell RK, and Lam K-W. Melanin may promote photoxidation of linoleic acid. Submitted to Biomedical Optics (Laser-Tissue Interaction VI)/Photonics West '95, to be held 4-10 Feb, 1995, San Jose, CA.

Stein CD, Glickman RD, Sowell RK, And Roach WP. Release of cytoplasmic lactate dehydrogenase (LDH) as an assay of laser tissue damage. Submitted to Biomedical Optics (Laser-Tissue Interaction VI)/Photonics West '95, to be held 4-10 Feb, 1995, San Jose, CA.

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